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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 23 October 1995 in connection with Application No. PN 6161 for a patent by LIONS EYE INSTITUTE filed on 23 October 1995.

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PRIORITY DOCUMENT

WITNESS my hand this Twenty-Eighth day of October 1996

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): LIONS EYE INSTITUTE

Invention Title: METHOD AND COMPOSITION FOR TREATMENT OF OCULAR DISEASES

The invention is described in the following statement:

METHOD AND COMPOSITION FOR TREATMENT OF OCULAR DISEASES.

This invention relates to a method and composition for treating ocular diseases, in particular retinal disease involving neovascularisation of the choroid and/or retina. It makes use of the phagocytic characteristic of specific cells in the eye to provide an effective manner of delivering an active agent to the target, for either short term or long term treatment of neovascularisation. The methods and compositions of the invention are useful for delivering DNA, RNA, anti-sense nucleotides, peptides or other therapeutic agents to phagocytic cells or surrounding cells.

BACKGROUND OF THE INVENTION

A variety of ocular diseases such as macular degeneration and diabetic retinopathy are characterised by neovascularisation of the choroid and/or retina. This process is the major cause of blindness in patients suffering from these conditions.

PRIOR ART TREATMENTS

In age-related macular degeneration (ARMD), the formation and haemorrhaging of a subretinal neovascular membrane (SRNVM) results in rapid and substantial loss of central vision. Various treatments which are available, but all are unreliable. Laser photocoagulation is the most acceptable type of treatment, but it still suffers from the disadvantages that damage by the laser rays causes dense, permanent scotoma (Schachet, 1994; Ibanez *et al*, 1995 and Hudson *et al*, 1995) resulting in temporary loss of vision, and inability to prevent progression of the condition in the long term because of recurrence of the neovascular membrane.

Thus this treatment provides an advantage only in terms of preventing *profound* visual loss.

Similarly, surgical removal of the SRNVM or of subretinal blood, or re-positioning of the fovea by rotating the retina have largely been unsuccessful, due to post-surgical complications and to minimum or temporary improvement in vision. These invasive forms of treatment and the corresponding complications therefore far outweigh the advantages gained, and are limited in usefulness.

Administration of Interferon α2a, which has some anti-angiogenic activity (Fung, 1991; Guyer *et al*, 1992 and Engler *et al*, 1994) and transplantation of retinal pigment epithelial (RPE) cells (Algvere *et al*, 1994) have also proved to be of limited usefulness, and initial promising results obtained with small groups of patients have not been confirmed in larger trials.

In addition to laser photocoagulation which, as described above, suffers from various disadvantages, the other main method of treating diabetic retinopathy is the control of blood glucose and blood pressure. The efficacy of such forms of treatment is limited by the motivation and compliance of the patient involved.

About 30% of the population above age 75 suffers from macular degeneration, and about 3 in 1000 individuals suffer from diabetic retinopathy. As each of these numbers will increase due to the aging of the population, and the increase in incidence of diabetes, there is a need for a more effective manner of treating these and other ocular diseases mediated by neovascularisation.

MECHANISM OF NEOVASCULARIZATION

Vascular endothelial cell growth factor (VEGF) is a dimeric, disulphide-bridged glycoprotein which is well-known to be synthesised and secreted by a variety of normal as well as tumour cells. Recent observations indicate that VEGF is frequently detected in the neovascular retinal membranes of patients with diabetes (Malecaze *et al*, 1994), the ocular fluid from patients with either diabetic

retinopathy or with central retinal vein occlusion (Aiello *et al*, 1994). More recently, it was found that VEGF expression was induced in conditions such as central vein occlusion, retinal detachment and intraocular tumours. In a rabbit model, levels of VEGF mRNA were elevated in the hypoxic region of the retina following induction of retinal vein occlusion. (Pe'er *et al*, 1995). Stimulation of VEGF expression by hypoxia has also been observed in other animal models (Pierce *et al*, 1995; Miller *et al*, 1994), and *in vitro* in all types of cell cultures (Simorre-Pinatel *et al*, 1994; Hata *et al*, 1995 and Thiema *et al*, 1995).

ANTI-SENSE DNA AND GENE THERAPY

The suppression of expression of genes encoding proteins which mediate undesirable activity has been achieved in a variety of situations by the introduction of 'anti-sense' DNA sequences into the DNA of target cells. These anti-sense sequences are DNA sequences which, when transcribed, results in synthesis of RNA whose sequence is antiparallel to the sequence encoding the protein. Such anti-sense sequences have been tested in a number of viral diseases. Alternatively, anti-sense oligodeoxynucleotides can be introduced into target cells; such short sequences are not themselves transcribed, but inhibit transcription and/or subsequent translation of the corresponding sense DNA sequence in the target cell.

In Australian Patent Application No. 75168/94 (HYBRIDON INC), it was shown that *in vitro* expression of murine VEGF could be inhibited in COS-1 or NB41 cells by incubation with 19- to 21-mer anti-sense oligonucleotides based on murine VEGF. A 21-mer antisense nucleotide targeted against the translational stop site was shown to be the effective sequence. There is no disclosure or suggestion of specific targeting of sequences to any tissue in the eye, or of treatment of any ocular conditions other than diabetic retinopathy.

In U.S. Patent No. 5,324,654, a method of stimulating proliferation of non-malignant cells is disclosed. The method comprises the *in vitro* treatment of cells with an anti-sense nucleotide corresponding to the retinoblastoma (Rb) gene to inhibit expression of the Rb gene product, resulting in suppression of the expression of proteins which inhibit cell growth. In this way, proliferation of cells is encouraged. The proliferated cells can then be re-implanted if desired, and the cells may be genetically engineered to replace a specific gene prior to re-implantation. However, there is no reference to use of this anti-sense sequence to treat conditions of the eye. The invention of US5324654 is directed to establishing cell lines capable of long-term proliferation and to treatment of conditions such as muscular dystrophy and diabetes, caused by failure to express a gene.

However, successful use of anti-sense nucleotides to counter expression of a gene *in vivo* is limited by factors such as the need for co-suppression of mutant gene expression (Milan, 1993; McInnes and Bascom, 1992), or the need for high concentrations of the anti-sense nucleotides (Akhtar and Ivinson, 1993).

Attempts to increase uptake of anti-sense sequences into the target cell by encapsulating these sequences in liposomes have been largely unsuccessful.

Another manner in which the targeting may be achieved is the employment of virus-mediated DNA transfer, using viruses such as the Sendai virus. Sendai virus is an RNA virus which has been shown to deliver DNA and proteins into cells with more than 95% efficiency (Kaneda *et al*, 1987). In this gene transfer system, DNA nuclear protein complex in liposomes is directly introduced into the cytoplasm of the cell by the fusion activity of Sendai virus. The DNA is delivered rapidly into the nucleus with nuclear protein. Sendai virus-mediated gene transfer occurs by fusion of the virus with the cell membrane, and

bypasses th endocytic pathway. Recently, highly efficient delivery of anti-sense or plasmid DNA into target cells by Sendai virus having been observed. Both the anti-sense and plasmid DNAs retained their activity not only in culture but also *in vivo* (Kaneda *et al*, 1987). However, the use of this virus is limited by the fact that there are no suitable constructs available at present to use as vectors. In addition, the transferred DNA can only be expressed for a limited period of time since the gene transfer is mediated by fusion.

Retroviruses have been widely used for somatic tissue gene therapy (Boris-Lawrie and Temin, 1993). They can target and infect a wide variety of host cells with high efficiency, and the transgene DNA integrates into the host genome. Theoretically, the integration of the DNA will provide permanent production of the transgene which could result in permanent rescue of the cells. However, retroviruses cannot infect non-dividing cells (Salmons and Günzburg, 1993). Furthermore, the retrovirus particles are unstable *in vivo*, which makes it difficult to achieve high virus titre with inoculation. In addition, there are significant concerns regarding the oncogenicity of the integrated viruses. The inability of retroviruses to infect non-dividing cells means that they cannot be selected as candidates for gene transfer in the eye, as the most important target cells such as photoreceptors and RPE cells are non-dividing cells.

The usefulness of herpes simplex virus vectors has been limited by their poor efficiency of infection (Culver *et al*, 1992). Two types of vectors have been developed, namely the replication defective recombinants and the plasmid-derived amplicons. The latter requires a helper virus. Although the toxic genes can be removed from the herpes simplex virus with difficulty, the constructs remain cytotoxic (Johnson *et al*, 1992). In addition, the long term expression of the sequences inserted has been

unsuccessful to date and there are problems with th regulation and stability of the constructs. The application of modified herpes simplex viruses to the eye in gene therapy poses major concerns because of their pathogenicity. Herpes zoster virus infection causes serious infections in the eye, frequently resulting in blindness requiring corneal transplantation.

Adenoviruses have been widely used for gene transfer in both non-dividing and proliferating cells. They can accommodate DNA up to 7.5 kb, and provide efficient transfection and high viral titre. The main advantage of using these rather than retroviruses is the ability to infect a wide range of non-dividing target cells (Kozarsky and Wilson, 1993). Replication-defective adenoviruses are considered to be relatively safe, in that these viruses are common pathogen in humans, usually causing relatively benign conditions such as colds. The vectors carry tumour genes with a deletion mutation, lowering the possibility of becoming oncogenic (Siegfried, 1993). In the first experimental gene therapy trial approved by the NIH (National Institutes of Health, USA) Recombinant DNA Advisory Committee, recombinant adenoviruses were used to treat individuals suffering from cystic fibrosis.

The main disadvantage of adenoviruses is their transient gene expression. Few attempts at gene delivery to non-dividing cells have been successful. The first successful gene transfer into the brain, which consists of non-dividing cells, was reported in 1993 using adenovirus s (Le Gal La Salle *et al*, 1993).

For treatment of ocular diseases, in which other sites in the body are largely or entirely unaffected, it is highly desirable to deliver the therapeutic agent, selectively to the target tissue in the eye. For anti-sense DNA, it is essential that the DNA be actually taken into these target cells.

These advances in gene therapy have led to further studies of the delivery and expression of transgenes into target cells such as beta-galactosidase transgene into the retina (Bennett *et al*, 1994, Li *et al*, 1994 and Mashmour *et al*, 1994) using recombinant adenovirus as a delivery system. High levels of transgene expression within 3 days in the RPE layer and within two weeks in the photoreceptor cells of the neural retina in young animals were observed. The expression of the reporter gene was followed up to 9 weeks. In older animals, neither subretinal nor intravitreal injections induced the expression of beta-galactosidase transgene in the photoreceptor cells (Li *et al*, 1994).

Australian Patent Application No. 61444/94 shows the uptake of replication-defective recombinant adenovirus by various tissues in the eye following injection into the anterior chamber, the vitreous humour, or the retrobulbar space, and that the reporter gene β-galactosidase is expressed. However, this document does not show that such forms of viruses successfully incorporate the active agent into the target cell or area. Nor is there any disclosure or suggestion that VEGF can be used to heal any ocular condition.

Another known obstacle to success of using anti-sense nucleotides as a form of therapy for the eye is the inability of the nucleotide to enter the target cells, and the limited stability of the oligonucleotides which have been modified, eg. phosphothioate oligonucleotides (Helene 1991). These factors greatly restrict the success of gene therapy *in vivo*, particularly in the long term. In the treatment of retinal diseases, the ability to delay progression of the conditions by about 12 months would greatly increase the value and effectiveness of long term therapy.

When using adenoviruses as a transport vector for retinal gene therapy, the associated cytotoxicity has been

shown to be dose-dependant (Mashmour, 1994). In ord r to decrease the dos of a given vector but retain its transfer efficiency, an adjuvant may be used. Adjuvants such as lipofectin have been shown to increase the uptake of "naked" DNA by cells.

HA is a large, complex oligosaccharide consisting of up to 50 000 pairs of the basic disaccharide glucuronic acid-β(1-3) N-acetylglucosamine β(1-4). It is found *in vivo* as a major component of the extracellular matrix. its tertiary structure is a random coil of about 50 nm in diameter. It has the ability to bind a large amount of water, which *in vivo* makes it a viscous hydrated gel with viscoelastic properties. It is found in this form in the mammalian eye, both in the vitreous and in the extracellular matrix.

The important characteristic of HA, in terms of its acting as a potential adjuvant is its ability to bind other molecules and to bind to cell membranes. Cell surface receptors specific to HA have been identified, including the histocompatibility antigen CD44 receptor for hyaluroan-mediated motility (RHAMM), intercellular adhesion factor (ICAM) and some homologous proteins in the CD44 family.

The putative adjuvant mechanism of action is based on the the presence of surface receptors on many cell types and the ability of HA to bind molecules. The binding of virus to the cell membrane facilitated by HA would allow the usual endocytotic mechanisms of viral uptake to be more effective.

Even though HA has been widely used in eye surgery as a replacement for vitreous humour lost during the surgical procedure, we are not aware of any suggestion in the art that HA promotes uptake of any pharmaceutical agent into any cells or tissues in the eye. Similarly, although HA has been suggested to promote penetration of pharmaceutical agents such as antibiotics or anti-cancer

agents, as set out in Australian Patent Application No. 52274/93 by Norpharmco, this specification does not suggest that HA promotes uptake of any agent, let alone DNA, or viruses by individual cells of any type. In particular, this invention does not teach the use of HA via intra-ocular injection.

The retinal pigment epithelium (RPE) is a non-renewable single cell layer in the eye, situated between the neural retina and the choroid. The cells of the RPE are phagocytic neuroepithelial cells which form the outer most layer of the retina. The phagocytic properties of these cells have long been known, and have been reviewed (Bok and Young, 1979).

We have now found that the phagocytic nature of the RPE cells enables the cells to take up large molecules such as oligonucleotides, following injection into the vitreous space *in vivo*, and that the use of replication-defective adenovirus to deliver the nucleotides confers stability to the latter. Our findings enable the induction of both long term and short-term inhibition of VEGF expression in retinal or choroid epithelial cells, and hence inhibition of neovascularisation of the retina.

SUMMARY OF THE INVENTION

According to one aspect, the invention relates to a pharmaceutical composition for treatment of retinal diseases mediated by abnormal vascularisation, comprising anti-sense nucleic acid sequences directed against vascular endothelial growth factor, and optionally further comprising adjuvants for increasing cellular uptake such as hyaluronic acid, together with a pharmaceutically acceptable carrier or vehicle.

In separate embodiments, this aspect of the invention is directed to treatment for such retinal disease in the short term (up to about two months), the long term (up to about one year), and indefinite (for the life of the

patient). In the first embodiment, for short term treatment the invention provides one or more anti-sense oligonucleotides having 100% complimentarity to a corresponding region of the VEGF gene. The oligonucleotide should have 16 to 50 nucleotides, preferably 16 to 22, and more preferably 16 to 19 nucleotides.

For long term inhibition, the invention provides a recombinant virus comprising VEGF DNA in the anti-sense direction. This VEGF DNA is a long sequence, which for purposes of this specification is to be understood to represent a VEGF sequence of greater than 50 nucleotides in length, ranging up to the full length sequence of VEGF. In this embodiment, the recombinant virus is accummulated in RPE cells, and produces anti-sense VEGF *in situ*, thereby inhibiting VEGF expression in the RPE cell. The VEGF is most preferably human retinal pigment epithelial (RPE) or choroidal endothelial VEGF.

In another aspect, the invention provides a method of prevention or amelioration of retinal diseases caused by abnormal neovascularisation, comprising the step of administering an effective amount of an anti-sense nucleic acid sequence directed against VEGF into the eye, thereby to inhibit neovascularisation.

For indefinite inhibition, the invention provides an adeno-associated or similar virus comprising VEGF DNA in the anti-sense direction. As in the embodiment directed for long term treatment, this VEGF DNA is of at least 50 nucleotides. The adeno-associated or similar virus facilitates integration of anti-sense VEGF DNA into the RPE cell genome, thus enabling expression of anti-sense VEGF for as long as the cell remains functional. Eye diseases which may be treated using the compositions and methods of the invention include but are not limited to age-related macular degeneration (ARMD) and diabetic retinopathy. Other ocular condition and tissues in which no vascularisation occurs (rubeosis iridis) or in the cornea

may also b treated by the invention.

The anti-sense sequence may be carried in a replication defective recombinant virus, as a vector or vehicle. The vector preferably comprises replication defective adenovirus carrying promotes such as the respiratory syncytial virus (RSV), cytomegalovirus (CMV), adenovirus major late protein (MLP), or β-actin promotes. In a particularly preferred embodiment, the vector is pAd.RSV or pAd.MLP. In a more particularly preferred embodiment the virus used is Ad.RSV.VEGF. The vector may also comprise a polyadenylation signal sequence such as the SV40 signal sequence.

In a preferred embodiment, human VEGF is subcloned into the vector in order to create the restriction sites necessary for insertion into the vector to form an adenovirus plasmid carrying VEGF on partial sequences thereof in an anti-sense direction, which can then be linearised by restriction enzyme digestion. The linearized plasmid which can then be co-transfected with a linearized replication defective adenovirus, such as Ad.RSV βgal recombinant virus in a suitable permissive host cell such as the kidney 293 cell line.

The compositions of the invention may be delivered into the eye by intra-vitreal or sub-retinal injection, preferably in an appropriate vechicle or carrier. Such methods of administration and vechicles or carriers for such injection are known in the art. Alternatively, *ex vivo* delivery in the compositions of the invention may be achieved by removal of RPE cells from the patient to be treated, culturing the cells and subjecting them to infection *in vitro* with a replication-defective adenovirus or an adeno-associated virus as defined above. RPE cells carrying the virus are then injecting into the sub-retinal layer of the eye of the patient.

While the invention is specifically described with reference to conditions of the eye, the person skilled

in the art will be aware that there ar many other pathological conditions in which VEGF is of importance. Such a person will understand that the antisense oligonucleotides and the recombinant viruses of the invention are applicable to treatment of such other conditions.

Brief Description of the Figures

Figure 1 shows the results of GeneScan analysis of persistance of anti-sense oligonucleotides *in vivo* in the retina following a single intra vitreal injection.

Figure 2 is a graphical representation of the number of phagosomes in the RPE layers of Long-Evans rats. Doses were as follows: Low 6.6 µg, medium 66 µg and high 132 µg of CatSC anti-sense oligonucleotide. Each column shows the mean and standard deviation of the number of phagosomes in five randomly selected areas in the rat retinas; and

Figure 3 is a graphical representation of the number of phagosomes in the RPE layers of RCS-rdy+ rats. Experimental animals were injected with 66 µg of sense oligonucleotides (S1) and 66 µg of antisense oligonucleotide (CatSC).

Description of the Invention

The invention will now be described by way of reference only to the following non-limiting examples. In some of these exaples, the feasibility of the methods utilised in the invention is demonstrated using anti-sense oligonucleotides complementary to cathepsin S (CATSC).

Example 1 Accumulation of Antisense Oligonucleotides in the RPE Cell Layer

Human retinal pigment epithelial cells were cultured and on the third passage were used for *in vitro* experiments. Confluent cultures were incubated with bovine

rod outer segments (ROS) to mimic the *in vivo* situation. A fluorescein-labelled anti-sense oligonucleotide complementary to human cathepsin S (CATSCF) was added to the medium of these cells and after 7 days of incubation, the cells were harvested. The presence of fluorescein-labelled oligonucleotides within the RPE cells was detected by fluorocytometry (FACS). A GeneScan DNA analyser was used to assess the presence and stability of the oligonucleotides in the cells. The fluorescence of cultured RPE cells was increased by about 100-fold, demonstrating the presence of the anti-sense oligonucleotides within the RPE cells. These results are summarised in Table 1

Table 1

Fluorocytometer measurements of human RPE cells incubated with or without complementary CATSCF

SAMPLE	FACS READINS
RPE + ROS	5.94
RPE + ROS + CATSC	8.50
RPE + ROS + CATSCF	461.50

It was not known if the fluorescence was emitted by the full length CATSC or by degraded oligonucleotides. Using GeneScan, it was demonstrated that the fluorescence was largely due to a 19-mer oligonucleotide, which appeared at a position similar to that of CATSCF. Using a similar procedure, it was observed that CATSC oligonucleotides were still intact after 7 days of incubation.

Example 2 Cellular Distribution of Oligonucleotides in Retinal Cells and Stability of Oligonucleotides Following Injection Into Eyes

One mmole of CATSCF was injected into the vitreous humour of 6-week old non-pigmented RCS-rdy$^+$ rats, and the movement of the oligonucleotides were followed by confocal fluoromicroscopy. Fluorescein (1mmole) was also injected as a control. Animals were euthanised 2 hours, 3 days and 7, 14 and 28 and 56 days after injection. Following euthanasia, the injected eyes were enucleated, frozen, sectioned and immediately used for confocal microscopy without fixation.

Two hours after intravitreal injection of CATSCF the penetration of the oligonucleotides were observed in the ganglion cell layer at 2 hours and also in the photoreceptor and pigment epithelial layers at 3 days. However, 7 days following injection, only the RPE layer had significant amounts of CATSCF. At 14, 28 and 56 days, a fluorescent signal was maintained in the RPE layer, and no signal was observed in any other cell types. These results show that a large proportion of CATSCF was taken up by the phagocytic RPE cells.

Following intravitreal injection as described above, eyes were dissected, the retina was removed, and the DNA extracted. The purified DNA was subject to GeneScan analysis. The presence of undegraded fluorescein-labelled oligonucleotide was demonstrated in the rat retinas after 7, 14, 28 and 56 days of injection, as shown in Figure 1. The intensity of the signal had significantly diminished by 56 days.

These results demonstrate that following intravitreal injection, oligonucleotides accumulate in the RPE cells. The oligonucleotides are present in the RPE layer up to 30 days and remain in a biologically active form during this period of time.

Example 3 Biological Activity of Anti-Sens Oligonucleotides

Female sixty day-old pigmented rats of the Long-Evans strain were obtained from Charles River Breeding Laboratories, Wilmington, MA.

Sixty day old non-pigmented RCS-rdy + rats were obtained from our colony. The animals were acclimatised to a 12 hr light/ 12 hr dark lighting cycle, with an average illuminance of 5 lux for at least 10 days prior to experimentation.

Animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Intravitreal injections through the pars plana were made using a 32 gauge needle. The left eyes served as controls, and the right eyes were injected with 3µl of 150 mM sodium chloride (saline), or with 3 µl of saline containing 6.6, 66 or 132 µg of CATSC respectively, an anti-sense oligonucleotide described earlier (Rakoczy *et al*, 1994) or 66 µg of sense oligonucleotide S1, 100% complementary to CATSC. Injected animals were allowed to recover from anaesthesia, and at one week post-injection were sacrificed by an overdose of sodium pentobarbital and used for morphological examination. All animals were killed within half an hour at the same time of the day, approx. 4 hours after light onset. Two to three animals were used for each dose.

Following enucleation, whole eyes were immersed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.125M sodium cacodylate buffer, pH 7.35. The cornea and lens were dissected free and the eyecup trimmed for orientation purposes. The tissue was fixed overnight at 4°C and then post-fixed for 1 hour in 1% osmium tetroxide at room temperature. After ethanol dehydration, the tissue was embedded in epoxy resin. Retinal sections were prepared for transmission electron microscopy (EM) as described previously (Kennedy *et al*, 1994).

Histological data were obtained by light microscopy. Semi-thin 1 μm sections were cut using a LKB 2088 Ultratome (LKB-Produkter, Sweden) with a diamond knife and stained with toluidine blue. The number of phagosomes that accumulated in the RPE cells of each specimen injected with saline, low (6.6 μg), medium (66 μg) or high 132 μg) dose of CATSC and 66 μg of S1 sense oligonucleotide was determined. From each eye, five sets of counts were made at 40 fold magnification and the standard deviation was calculated. Each set consisted of the total number of phagosomes in 250 μm length of RPE from 6 different randomly selected areas. The number of phagosomes that accumulated in the RPE of the control eyes, low medium and high doses of CATSC were analysed and graphically represented. Comparisons were made using the analysis of variance following the general linear models procedure of the SAS^R (version 6) statistical package (SAS Institute Inc., USA).

The results show that we successfully tested an antisense oligonucleotide (CATSC) in two strains of rats. The number of phagosome-like inclusion bodies present in control Long-Evans and RCS rdy + rats was not significantly different, 35.8+11.6 and 47.29+14.8 (mean ± SD), respectively, The intravitreal injection was non-traumatic. Light microscopic examination of the retinas of the saline injected eyes revealed no damage to the outer layers of the retina, and there was no increase in the number of phagosome-like inclusion bodies in the RPE layer when compared to the control non-injected animals. Long-Evans rats were used to identify the minimum amount of CATSC required to induce biological changes in the RPE layer. In the control eyes and in those injected with low dose (6.6 μg) of CATSC, the number of phagosome-like inclusions within the RPE cells, were 35.8+11.6 and 35.0+7.4 respectively. In animals injected with higher doses (66μg and 132 μg), the number of phagosome-like

inclusions were 96.2+13.6 and 141.0+34.7, respectively, and the difference was statistically significant when compared to the control and low dose samples (Figure 2).

RCS-rdy+ rats injected with 66 µg of CATSC also demonstrated a statistically significant increase in the number of phagosome-like inclusion bodies, ie 204.20+39.3 when compared to the 47.20+14.8 in controls. In contrast, the injection of 66 µg of sense oligonucleotide (S1) did not increase the number of phagosomes (Figure 3) present in the RPE Layer, (34.4+12.54).

The inclusions found in RPEs of CATSC-injected Long-Evans and RCS-rdy+ animals were spherical in shape, and clearly distinguishable from the very dark, small elliptical melanin granules present in Long Evans rats. In the presence of 66 µg of CATSC, the tips of the outer segments showed signs of disorganisation and there were some vacuoles present in the outer nuclear layer. However these changes were not observed in S1 sense oligonucleotide-injected animals.

Electron microscopic examination of the RPE layer of a CAT SC-injected eye revealed no significant changes in the morphology of RPE cells. Melanin granules appeared smaller and less concentrated due to regional differences. Individual mitochondrial profiles were smaller in the treated group than in the controls, although the number was greater in the treated than in the untreated animals. Electron microscopic examination confirmed that the structures of the undigested material was similar to that of phagosomes (1,2,3). The numerous phagosomes seen in the RPE layer of rats treated with CATSC were paranuclear, and contained mainly compacted phosholipid membranes, resembling undigested photoreceptor outer segment (POS) and confirming their photoreceptor origin. There were no other morphological changes observed in the POS layer, except for the disorganised appearance of the apices in treated animals.

Example 4 Delivery of Vehicle Molecules to the RPE Cell Layer

The effect of hyaluronic acid as adjuvant

However, we decided to study the effects of hyaluronic acid (HA) on viral gene transfer and its ability to stimulate uptake of DNA transported in an adenoviral vector were examined.

1% Hyaluronic acid extracted from rooster combs (HEALON, Pharmacia) was prepared as 10 mg/ml solution. For all experiments, HA was diluted with MEM. The moiety whose uptake of which was to be measured using HA as adjuvant was Ad.RSV.βGal

The effect of chondroitin sulphate and lipofectamine on the uptake of the above compounds was also investigated.

RPE or F2000 fibroblast cell cultures were grown as described in (Rakoczy *et al*, 1992). They were harvested and pooled. One ml aliquots of the pooled cell suspension were placed into each well of a 24 well plate, to ensure equal seeding of wells. The cells were incubated and allowed to reach 95% confluency.

Solutions of 0.001% and 0.002% HA were prepared using MEM.10 µl of virus solution was added to 10 ml of each of the diluted HA solutions and to 10ml of MEM control, and incubated for 30 minutes at 25°C with intermittent gentle shaking.

One ml of each of the test solutions and control was added to each cell of a 24 well-plate. There were four samples for each of the test concentration and the control. The viral solutions were incubated with the cells for 16 hours. Each experiment was terminated by removing the medium from the well and fixing the cells with 0.5 ml of 0.5% glutaraldehyde per well. The glutaraldehyde was removed after 5 minutes and the cells were washed once with PBS. Following this, 0.5 ml of x-gal stain (Australan Biosearch, Perth) was added to each well and incubated

overnight at room temperature.

Counting was carried out by a single observer. A second observer then blind counted half of the plates as a countercheck.

All cells staining positively with the x-gal stain were counted. For F2000 fibroblasts, the entire plate was counted at 200X magnification using a counting graticule within the microscope. For the RPE7 experiments, half of each plate was counted and the results multiplied by 2.

The following doses of adjuvants were used:-

	0.05%	0.01%	0.005%	0.001%	Control	Control
5 µl	176[a]	318	319	316	279	282
10 µl	305[a]	906	802	645	623	609
25 µl	-[a]	714[b]	1682	1822	1478	1184
50 µl	-[a]	2772	2692	3328	2250	1822

[a] The viscosity of these solutions precluded adequate dispersion of the HA and made them very difficult to manipulate.

[b] It was not clear why this figure fell so far outside of the normal distribution of the other results.

Effect of HA as an Adjuvant on the uptake and expression of the β-gal gene using a viral vector

Experiment 1

	1	2	3	4	Average
RPE 7/HA (0.001%)	17114	20776	18730	17998	19168
RPE7/HA (0.005%)	17688	22186	20258	22236	20592
RPE 7/Cont	10782	15480	16326	15266	14705

Experiment 2

	1	2	3	4	Average
F 2000/HA (0.001%	4358	4620	Contam.	?	4489
F 2000/HA (0.005%)	4506	3914	4759	4332	4378
F2000 Cont	3884	3652	3875	3748	3789

The two-tailed student *t* Test was used to assess the significance of the difference between the means for each set of experimental data. For each experiment, the means, the Standard error of the differences of the means and the p value for the t Test are given. In both experiments, HA gave very significantly increased uptake ($p < 0.05$).

Example 5 Up and Down Regulation of Cathepsin D Expression in NIH 3T3 Cells

A 1620 bp HindIII fragment of human cathepsin D was subcloned into pHBApr-1-neo vector in both sense and anti-sense directions. Positive clones were selected, and

the orientation of the fragments was confirmed by EcoRI restriction enzyme analysis. For the transfections of NIH 3T3 cells the clones carrying cathepsin D in anti-sense and sense direction were on caesium chloride density gradients.

NIH 3T3 cells were seeded onto 6 well tissues culture plates at a concentration of $2X10^5$ in 2 ml DMEM supplemented with 10% fetal bovine serum (FBS). The cells were incubated overnight at 37°C until they became 70% confluent. Having reached this confluency the cells were washed twice with serum and antibiotic-free medium. Lipofection reagent (10 µl) (GIBCO-BRL) and 2 diluted in 100 µl of OPTI-MEM (GIBCO-BRL) were gently mixed and incubated at room temperature for 15 minutes. Following incubation an additional 800 µl of OPTI-MEM was added to the mixture. This diluted mixture was gently overlaid onto the washed NIH 3T3 cells. The cells were incubated for 16-20 hrs before the transfection media was removed and replaced with DMEM supplemented with 10% FBS. After a further 48 hrs incubation the cells were trypsinised and subcultured at 1:5 in media containing 10% FBS and Geneticin 418 (GIBCO-BRL) at 1 ng/ml concentration. Successfully transfected cells selected with Geneticin 418 were maintained in media supplemented with BS and Geneticin 418 as described earlier. Confluent transformed cultures were frozen for storage and subcultured for further analysis. The presence of cathepsin D in the transformed NIH 3T3 cells were detected with polyclonal antibody against cathepsin D, using a conventional cytochemical technique using an alkaline phosphatase-labelled second antibody.

Results

The presence of cathepsin D fragment of the vector was demonstrated with HindIII digestion Positive clones demonstrated the presence of 1620 kb fragment. The

orientation was established by ECO RI restriction enzyme digestion, which gave two fragments at 5.7 and 5.9 kb in the case of anti-sense orientation and 4.3 and 7.3 kb in the case of sense orientation. All NIH 3T3 cells surviving Geneticin 418 selection carried cathepsin D clones, which carry antibiotic resistance. The transformed control NIH 3T3 cells did not survive the selection procedure. Immunocytochemistry results suggest that NIH 3T3 cells carrying cathepsin D in the sense direction unregulated cathepsin D production, while those carrying cathepsin D in the anti-sense direction down regulated cathepsin D production.

Example 6 Cloning and Characterisation of Human RPE Vascular Endothelial Growth Factor (RPE-VEGF)

Human RPE cells, available in our laboratory, are grown in tissue culture. To upregulate VEGF expression cell cultures are treated in hypoxic conditions. The upregulation of VEGF expression is monitored with immunohistochemistry. The mRNA is extracted from 10^7 RPE cells, and a cDNA library carrying all genes expressed in the RPE/choroid is established using methods known in the art.

VEGF is a highly conserved molecule which is highly homology between different species. A murine VEGF cDNA clone, available in our laboratory, is used to screen the human RPE cDNA library in order to identify the full length human RPE-VEGF clone. Positive clones are analysed by restriction enzyme analysis and finally by DNA sequencing. Full length RPE-VEGF clones are analysed to elucidate their genomic structure (initiation sequences, start and stop codons, putative exons *etc.*).

Clones carrying the full length RPE-VEGF are analysed for the expression of VEGF protein with *in vitro* translation. The identified clones are used to derive the

anti-sense molecule for insertion into the vehicle system and for the selection of the anti-sense oligonucleotides.

Example 7 Pharmaceutical Agent for the Short Term Inhibition of VEGF Expression

Anti-sense DNA technology enables the sequence specific inhibition of target molecules without affecting the non-targeted functions of the cell. As described above, we have demonstrated both *in vitro* and *in vivo* that anti-sense DNA can be used effectively to inhibit the anti-sense oligonucleotide into the vitreous.

A panel of 16-19-mer oligonucleotides, 100% complementary to parts of the VEGF gene, is selected from the 5' and 3' ends of the DNA sequence. Sense and scrambled sequences are also used as control. Phosphothioate-protected oligonucleotides are sythnesized on a DNA synthesizer and subjected to purification.

Example 8 Anti-Sense Agent for the Long Term Inhibition of VEGF Production

Human VEGF is subcloned into the appropriate vectors in order to create the restriction sites necessary for insertion into p.Ad.RSV. The adenovirus plasmid containing the human RPE-VEGF gene in the anti-sense direction is characterised and positives are subjected to homologous recombination.

The recombinant plasmid pAd.RSV.a.VEGF is linearised Ad.RSV.bGal recombinant virus. Confluent layers of kidney 293 cells are co-transfected with the linearised plasmids and incubated by 7 days.

Using blue-white colony selection white colonies are picked and analysed for the presence of VEGF in anti-sense orientation. Initially the presence of VEGF anti-sense oligonucleotide is identified by restriction enzyme analysis, PCR amplification followed by Northern blot analysis looking for the expression of anti-sense VEGF RNA.

Modification of the recombinant adenovirus are performed to assess their effect on improving anti-sense VEGF DNA expression. These include removal of polyadenylation signal, change of promoter, shortening the VEGF insert.

Example 9 Construction of a Vehicle for the Permanent Expression of Target Molecules

The vehicle described in Example 8 is suitable for long term treatment in that it provides temporary (maximum one year) expression of the anti-sense VEGF DNA molecule and consequent protection against neovascularisation. To achieve indefinite treatment, we propose the application of a vector system which enables the integration of VEGF in anti-sense direction into the human genome present in RPE cells, which means that the protection against neovascularisation can be provided for the rest of the life of the patient (as long as the RPE cells remain functional), using an adeno-associated virus (AAV) vector.

Adeno-associated viruses are non pathogenic, are able to infect non-dividing cells, an have a high frequency of integration. We use AAV-2, which is a replication defective parvovirus which can readily infect other (RPE) cells and integrate into the genome of the host cells. Recent characterisation has revealed that it specifically targets the long arm of human chromosome 19.

AAV constructs will use varying promoter sequences in combination with a reporter gene. The expression of the reporter gene mRNA may be detected with PCR amplification or *in situ* PCR, and the integration of the reporter gene may be identified by chromosomal analysis of RPE cells.

Using the appropriate restriction sites the reporter gene is replaced by anti-sense VEGF DNA is cloned. The new construct is co-transfected with he complementing plasmid (pAAV/ad) into kidney 293 cells previously infected

with adenovirus type 5 to make rAAVaVEGF construct. The construct produced is used to infect RPE cells, and the expression of anti-sense VEGF is detected by PCR amplification.

Example 10 Model Systems for Testing Inhibition *In Vitro*

Human VEGF is cloned into COS cells to produce a culture system (VEGF-COS) in which the effective inhibition of VEGF expression can be tested. The inhibition of VEGF expression is tested by Northern and Western blot analyses and quantified by immunoassay.

Toxicity of increasing concentration of oligonucleotides on COS cells is assessed with the trypan blue assay. The proliferation of COS cells is monitored with or without the presence of increasing concentration of oligonucleotides. The inhibition of the expression of VEGF in controls and in cultures maintained in the presence of anti-sense oligonucleotides is monitored by Northern and Western blot analyses, immunocytochemistry and by a quantitative immunoassay.

RPE cells are cultured in hypoxic conditions and the upregulation of VEGF expression are monitored in the presence of increasing concentration of oligonucleotides for an extended period of time. Toxicity, proliferation assay and the monitoring of VEGF expression are performed as described earlier.

CEC cells are cultured in normal and hypoxic conditions wit or without the presence of increasing concentration of oligonucleotides. In addition to the toxicity, proliferation assay and VEGF detection the affect of anti-sense oligonucleotide mediated inhibition of VEGF expression on tube formation is analysed. RPE/CEC dual cultures produced in normal and hypoxic conditions will be subjected to similar tests. The same model system are used to ass ss the long term and permanent agents of the

invention.

Example 11 Inhibition of RPE-VEGF Expression with Anti-Sense Oligonucleotides, Ad.RSV.aVEGF and rAAVaVEGF *In Vivo* in Rats

Neovascularisation can be induced using pocket implants in the choroid or the subretinal layer. One of the disadvantages of these models is that the process of neovascularisation might not follow the same biochemical steps which naturally occur in humans suffering from ARMD. To overcome these difficulties we use an animal model in which choroidal neovascularisation is induced by VEGF overexpression in the RPE cells. Using recombinant adenoviruses carrying VEGF, for example Ad.RSV.VEGF, for the *in vivro* trials all animal models described above are utilised to provide us with a wide range of information. Tests are conducted to demonstrate the expression of a VEGF expression over a period of one year. Using Northern and Western blot analysis VEGF down-regulation is monitored and immunohistochemistry is used to demonstrate the down-regulation of VEGF expression in a cell-specific manner. Using the above described animal models, choroidal neovascularisation is monitored by histology and angiography. These models are applicable to all these embodiments of the invention.

It will be apparent to the person skilled in the art that while the invention has been described in the Examples, various modifications and alterations to the embodiments described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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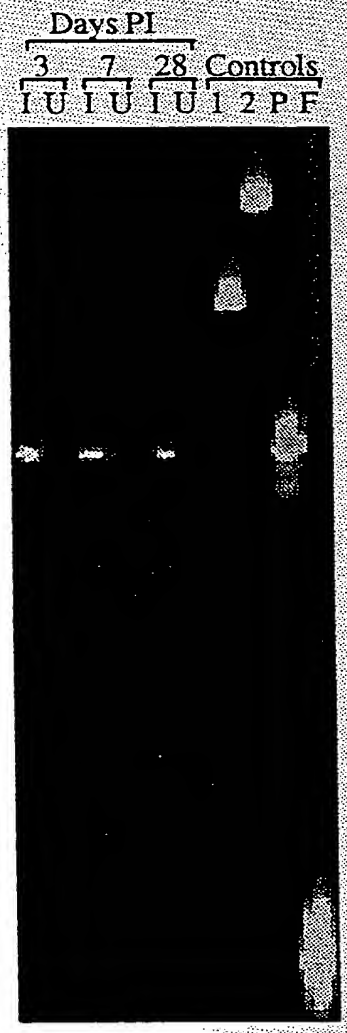
Antisense Oligo Persistence In The Retina / RPE of Rat Eye Tissue



1= FAM 23 bases; 2= FAM 27 bases;
P= Antisense Primer; F= FAM Dye
I= Injected oligo; U= Uninjected.

Fig. 1

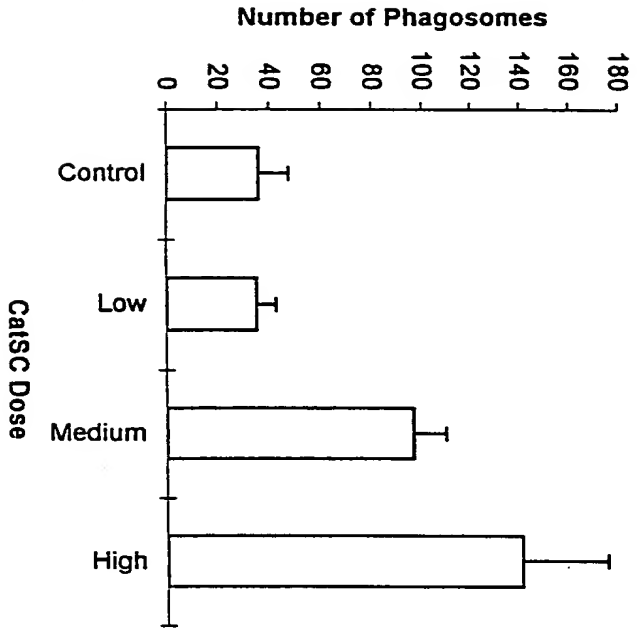
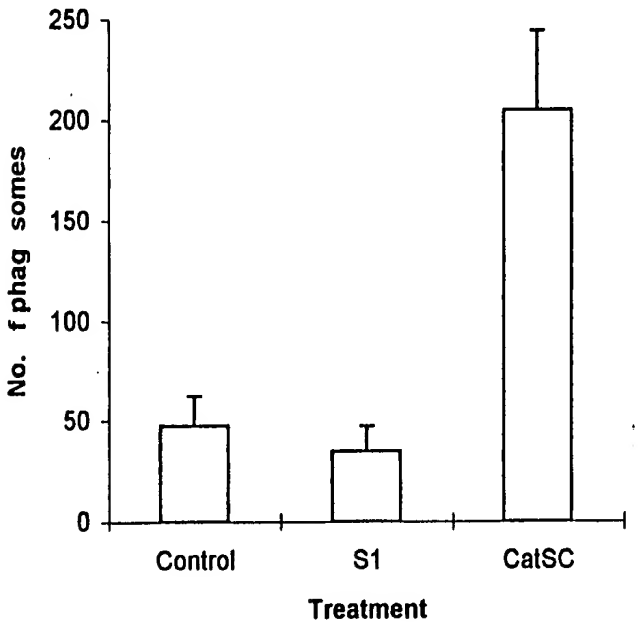

Number of Phagosomes
0
20
40
60
80
100
120
140
160
180
Control
Low
Medium
High
CatSC Dose

Fig. 2

Fig. 3


250
200
150
100
50
0
No. f phag somes
Control
S1
CatSC
Treatment